Supplementary Data

Gene expression profiling of hypertrophic cardiomyocytes identifies new players in pathological remodeling

Short title: Transcriptome of failing cardiomyocytes

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Supplementary Figure and Table legends

Online supplementary Figure S1. Time-course analysis after pressure overload to identify adaptive and pathological hypertrophic stages. A, Schematic of transverse aortic banding (TAB) time-course study. **B**, Aortic pressure gradient determined by echocardiography (n=7-8). **C**, Representative images of H&E, wheat germ agglutinin (WGA, middle row) and Picrosirius red (SR, bottom row) stained cardiac sections. Scale bars represent 200μm or 50μm. **D**, Heart weight to tibia length (HW/TL) ratios (n=7-8). **E**, Quantification of cross-sectional area (CSA) of CMs (~80 cells per animal, n= 3-4 per group). **F**, Quantification of ventricular fibrosis (n=2-4 per group). **G**, Real-time PCR analysis of *Nppa, Myh7, Myh6*, *Col3a1, Pgc1*α and *Pgc1*β on cardiac tissue collected at the indicated timepoints after TAB (n=3-4). Data expressed as mean fold change ±SEM; *indicates $p<0.05$ compared to sham (S) in a One-way ANOVA or unpaired t-test.

Online supplementary Figure S2. Fluorescence-activated cell sorting (FACS) strategy for enriching for CMs and RNA quality from sorted CMs. A, FACS plots showing the gating strategy to distinguish tdTomato positive CMs from tdTomato negative nonCMs in *Cre* negative (upper row) and *Myh6-Cre;tdTomato* positive (lower row) hearts. **B**, Histogram showing size distribution of sorted CM ($DAPI^-$ $tdTomato^+$ 488^{auto} $FCSW^{high}$) and nonCM ($DAPI^-$ $tdTom^-$) based on forward scatter width (FSC-W) **C**, Bioanalyzer plot of RNA isolated from increasing amounts of sorted CMs in Sham conditions, and their respective RNA integrity number stated below**. D,** Bioanalyzer plot of RNA isolated from 50,000 sorted CMs (CMs) and 50,000 sorted cells that are not selected with the cardiomyocyte gating strategy, mostly non-CMs (NCMs).

Online supplementary Figure S3. **Downregulated genes in hypertrophic or pathological CMs.** List of genes downregulated in hypertrophic and/or failing CMs. **A,** List of the top 30 downregulated genes in one-week TAB CMs (log2FC > 1). **B,** List of all the genes (22 genes) that are downregulated in one and eight-week TAB CMs (log2FC < -1). **C,** List of the top 30 downregulated genes in eight-week TAB CMs (log2FC > 1).

Online supplementary Figure S4. List of mitochondrial genes in hypertrophic and pathological CMs. Regulation of all mitochondrial genes detected in one and eight-week TAB CMs.

Online supplementary Figure S5. Single-cell sequencing analysis on sham and post-IR hearts to identify fibroblast marker changes (n=3)**. A,** t-SNE map indicating transcriptome similarities among all individual cells. Different numbers and colors highlight the different clusters. **B,** t-SNE map indicating the origin of fibroblast and cardiomyocyte population: fibroblasts from sham (light blue) and post-IR (dark blue) hearts, and CMs from sham (light red) and post-IR (dark red) hearts. **C,** t-SNE map indicating the expression of *Col3a1* in cell populations indicated as fibroblasts and CMs. **D,** Scatter plot showing the expression changes of well stablished fibroblast markers in the fibroblast population (blue) and in the cardiomyocyte population (red). Data obtained from single-cell RNA sequencing data set, and differences are based on base-mean reads across all conditions and Log2 fold change.

Online supplementary Figure S6. hiPSC-derived CM purity after differentiation. FACS plots representing the % of CMs before and after lactate selection based on cardiac troponin T (cTnT) expression.

Online supplementary Table S1. Morphological measurements from control (Sham) and transverse aortic banding (TAB)-operated mice after 1, 2, 4, 6, or 8 weeks (w) of TAB.

Heart weight (HW), bodyweight (BW), tibia length (TL) are shown, as HW, Lung weight (Lu) and liver weight (Li) normalized to BW or TL. Average ± SD is shown. * indicates $p<0.05$ compared to Sham with One-way ANOVA test (n=7-8).

Online supplementary Table S2. Primer pairs used in real-time PCRs to quantify mRNA.

Online supplementary Table S3. In Situ Hibridization primers for probes.

Supplementary materials and methods

Animals

Mice were maintained on a C57Bl/6J background (stock number 000663, Charles River).

Tissue collection

For cardiac tissue collection, mice were euthanized by cervical dislocation. The chest was opened to expose the heart. The heart was removed, washed in ice-cold PBS and weighed. For protein/RNA analysis, the heart was snap-frozen in liquid nitrogen. For cardiomyocyte sorting, the atria were removed and the heart was immediately perfused by injection of 10ml of cold perfusion buffer into the left ventricle. After perfusion, the heart was removed and washed in cold perfusion buffer.

Perfusion buffer: 135mM NaCl, 4mM KCl, 1mM MgCl2, 10mM HEPES, 0,33 mM NaH2PO4, 10mM glucose, 10mM 2,3-butanedione monoxime (Sigma, St Louis, MO), 5mM taurine (Sigma), adjust pH to 7,2 at 37°C.

Perfused hearts were used for digestion. The left leg was collected, enzymatically digested with ProtK (Promega, 0.6µg/µl final concentration) in lysis buffer, and tibia length was measured with a digital caliper.

Imaging of the cells

After digestion of the heart 5000 cells were sorted into 96 wells plate and imaged using EVOS Cell Imaging Systems to visualize the morphology of the digested or sorted cells.

RNA isolation and quality control

The RNA concentration was assessed by a NanoDrop Spectrophotometer. To assess the RNA quality, an aliquot of RNA was diluted to 200-5000pg/µl and put on a bioanalyzer using Agilent RNA 6000 Pico Kit according to the manufacturer's instructions.

RNA sequencing of sorted CMs

Primers consisted of a 24 bp polyT stretch, a 4bp random molecular barcode, a cell-specific 6bp barcode, the 5′ Illumina TruSeq small RNA kit adaptor and a T7 promoter. After addition of these CELseq primers, mRNA was then reverse transcribed, converted to double-stranded cDNA, pooled and *in vitro* transcribed for linear following the CEL-Seq 2 protocol. The aRNA that resulted from the IVT was then again reverse transcribed using a Random Hexamer primer as described in the CELseq2 protocol. From this cDNA, Illumina sequencing libraries were then prepared with the TruSeq small RNA primers (Illumina) and sequenced paired-end at 75 bp read length the Illumina NextSeq500.

Data analysis of cardiomyocyte RNA-sequencing

Paired-end reads from Illumina sequencing were mapped with BWA-ALN to the reference genome *M. musculus* GRCm38 (version 0.7.10). Transcript count for every gene was recorded. Differential gene expression between groups was calculated using the DESeq2 package (1). Up- and downregulated genes were identified as genes that changed 2-fold (up or down) in TAB animals compared to sham animals at the same timepoint. Lists of up- or downregulated genes were submitted to the online database for annotation, visualization and integrated discovery (DAVID) (2)·(3) to identify enriched GO-terms in these differentially expressed genes.

Data accessibility

RNA-Seq data has been made publicly available through the NCBI Gene Expression Omnibus (GEO), GEO accession number GSE138299.

Quantitative real-time PCR

Transcript quantities were normalized for endogenous loading. Fold changes in gene expression were calculated according to the $2^{-\Delta\Delta CT}$-method and expressed as mean fold change ± SEM. The qPCR primers used can be found in online supplementary Table S2.

Histology, immunohistochemistry and in situ hybridisation

Adult hearts were excised from euthanized mice, washed in cold PBS and fixed with 4% formalin at room temperature for 48h, embedded in paraffin and sectioned at 4μm. Cultured CMs on cover-slips were washed with PBS and fixed with 4% formaldehyde at room temperature for 20 minutes. Sections were used for:

Hematoxylin and eosin (H&E) and picrosirius red (SR) staining using standard procedures. 4-chamber views or short-axis cross-section view and high magnification images were made using a Leica DM 4000 microscope and Leica LAS software. Fibrotic areas in the ventricles were quantified using ImageJ 1.49v software. Quantifications were based on 2-7 hearts per experimental group.

WGA-staining. Images were generated using a Leica TCS SPE and Leica LAS software. Cross-sectional area (CSA) was measured using ImageJ 1.49v software. Quantifications were based on >50 cells per heart and 5-7 hearts per experimental group.

Immunohistochemistry. The images were taken using the Leica TCS SPE confocal microscope. Antibodies used include mouse anti-α actinin (ACTN2, Sigma-Aldrich, #A7732) and goat anti-tdTomato (Sicgen, AB8181-200) to visualize CMs. We used corresponding secondary fluorescent antibody anti-mouse Alexa-488 and anti-goat Alexa-568 (Life Technologies).

In situ hybridization. The slides were prepared for hybridization by the following incubations: 0.2M HCl for 15 minutes; 30μg/ml Proteinase K for 15 minutes. Slides were rinsed with 0.2% glycine to stop Proteinase K reaction. The slides were post-fixed with 4% paraformaldehyde for 10 minutes, then in acetic anhydride for 5 minutes twice, and washed with SSC buffer. Prehybridization was done for 1 hour at 65°C. Probes were used 1μg/ml in hybridization solution and denatured by heating at 95°C for 2 minutes and followed by cooling on ice. Hybridization was done for 24 hours at 62°C. After removal of hybridization buffer, the slides were incubated with 50% formamide/2xSSC buffer at 60°C for 15 minutes three times. Slides were washed by TBS-T and maleic acid buffer and subsequently blocked by 1:1500 anti-DIG-AP in blocking solution for 3-4hrs at room temperature. Slides were washed in maleic acid, TBS-T and NTM buffers. Probe detection was carried out by incubation in NBT/BCIP

substrate for 18-24 hr in the dark. The detection reaction was stopped by washing the slides in demi-water, after which they were mounted with aquatex.

Human Heart Samples

Human RNA sequencing: After the selection of mRNA, libraries were prepared using the NEXTflex™ Rapid RNA-Seq Kit (Bio Scientific). Libraries were sequenced on the Nextseq500 platform (Illumina), producing single-end reads of 75bp. Reads were aligned to the human reference genome GRCh37 using STAR v2.4.2a (4). Picard's AddOrReplaceReadGroups v1.98 (http://broadinstitute.github.io/picard/) was used to add read groups to the BAM files, which were sorted with Sambamba v0.4.5 (5) and transcript abundances were quantified with HTSeq-count v0.6.1p1 (6) using the union mode. Subsequently, reads per kilobase million reads sequenced (RPKMs) were calculated with edgeR's RPKM function (7). To obtain a list of differentially expressed genes between HCM and controls at FDR<0.05, we employed Deseq2 using Galaxy (8). The p-values were calculated using Wald statistics and corrected for multiple testing using the Benjamini-Hochberg method. Gene expression values obtained by RNA sequencing were plotted for regulation analysis and correlation analysis.

Human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes

Differentiation was induced with differentiation medium (Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies), 0.5 mg/ml Albumin (human recombinant, Sigma-Aldrich), 0.2mg/ml L-Ascorbic Acid 2-phophate (Sigma-Aldrich)) with freshly added GSK3B-inhibitor CHIR99021 (4μM, Millipore). This factor was removed at day 2 and differentiation medium was added including a Wnt-inhibitor (5μM IWP2, Millipore). At day 4 and 6 the cells received new differentiation medium without additions. Finally, at day 8 (and every 2/3 days) medium was refreshed with cardiomyocyte culture medium (RPMI 16 40 with HEPES with GlutaMax, plus 20μL/mL B27 supplement (both Life Technologies).

Intracellular calcium transients

Cells were recorded at 37°C in Tyrode's solution (5mM KCl, 140mM NaCl, 1mM MgCl2, 1.8mM CaCl2, 10mM HEPES, 10mM glucose, adjusted pH to 7.4 using NaOH). The recordings were all baseline measurements using software Micro-Manager, version 1.4.23, along with ImageJ.

ELISA assay

The supernatant of a million hiPSC-CMs per well in a Geltrex coated 6-well plate exposed to culture medium with or without addition of 10μM final concentration L-Norepinephrine (NE, Sigma-Aldrich) and 1μM final concentration of Angiotensin II (AngII, Sigma-Aldrich) was collected every 2/3 days. The NT pro-BNP kit (R&D systems, USA) was used to measure secreted NT pro-BNP levels according to manufacturer's instructions.

Primary cardiomyocyte culture

Primary cardiomyocytes were initially maintained in Ham's F10 medium (Gibco) supplemented with 5% FBS (Sigma-Aldrich) and 1% penicillin/streptomycin (Life Technologies). The day after isolation, cardiomyocytes were switched to serum-free Ham's F10 medium, supplemented with 1% penicillin/streptomycin and 1μl/ml insulin-transferrin-sodium-selenite supplement (Sigma-Aldrich, catalog number 11074547001).

Online supplementary Table S2. Morphological data showing increased heart weight in TAB compared to Sham in wild type mice.

	Sham (n=7)	**1w TAB** (n=8)	**2w TAB** (n=7)	**4w TAB** (n=8)	**6w TAB** (n=7)	**8w TAB** (n=7)
HW (mg)	142.54 ±14.91	217.61 ±35.99 *	184.23 ±24.83 *	228.14 ±37.00 *	294.41 ±33.28 *	239.91 ±43.60 *
BW (g)	27.76 ±0.99	26.51 ±0.80 *	25.56 ±1.10 *	27.80 ±1.42	31.03 ±1.67 *	30.57 ±1.43 *
TL (mm)	16.66 ±0.10	16.44 ±0.18 *	16.40 ±0.18 *	16.73 ±0.13	17.10 ±0.26 *	16.91 ±0.22 *
HW/BW (mg/g)	5.13 ±0.46	8.23 ±1.48 *	7.23 ±1.09 *	8.11 ±1.26 *	9.47 ±0.70 *	7.83 ±1.26 *
Lu/BW (mg/g)	6.36 ±0.42	6.96 ±0.41 *	7.33 ±1.33	9.04 ±1.40 *	6.50 ±0.88	6.39 ±0.91
Li/BW (mg/g)	53.68 ±1.46	58.63 ±1.87 *	50.07 ± 2.76 *	52.87 ±2.84	52.12 ±1.85	50.20 ±2.82 *
HW/TL (mg/mm)	8.56 ±0.92	13.22 ±2.11 *	11.23 ±1.49 *	12.76 ±2.73 *	17.22 ±2.01 *	14.17 ±2.44 *
LU/TL (MG/MM)	10.58 ±0.59	11.13 ± 0.70	11.42 ±2.10	17.14 ±6.40 *	11.75 ±1.39	11.53 ±1.55
LI/TL (MG/MM)	89.45 ±4.50	94.69 ±4.00 *	78.10 ±6.31 *	89.06 ±8.93	94.56 ±5.94	90.86 ±7.85

Online supplementary Table S2. Primers qPCRs

Gene	Species	Forward primer	Reverse primer
Actn2	mouse (mmu)	agatgaccctgggtatgatctg	tgtatggagctgtcttcctctg
Cdk1	mouse (mmu)	aagtgtggccagaagtcgag	tgagagcaaatccaagccgt
Col3a1	mouse (mmu)	gatggcaaagatggatcacctgg	gacccttttctcctgggatgc
Gapdh	mouse (mmu)	tgtcgtggagtctactggtg	acacccatcacaaacatgg
Myh6	mouse (mmu)	gttaaggccaaggtcgtgtc	gccatgtcctcgatcttgtc
Myh7	mouse (mmu)	tgacgcaggagagcatcat	gagtgcatttaactcaaagtccttc
Nppa	mouse (mmu)	aggccatattggagcaaatc	cctcatcttctaccggcatc
Nppb	mouse (mmu)	gagtccttcggtctcaaggc	caacttcagtgcgttacagc
Pgc1α	mouse (mmu)	tcaagaacgaaagtcggagg	ggacatctaagggcatcac
Pgc1β	mouse (mmu)	ggccttgtgtcaaggtggat	gcaccgaagtgaggtgctta
AKAP2	human (hsa)	ggaggctaccactccctgc	gagggatggtccatgggatg
ANKRD23	human (hsa)	ggaccatggacttcatcagcattcagca	tcagcaccgggtgcggggatgcgccacgt
CLIC5	human (hsa)	cccaaactggctgcaaaaca	taggcctctttcaagagcagc
EFDH2	human (hsa)	gacggcttcatcgacctgat	gacgtcgatctcagagaggc
KCNJ2	human (hsa)	accgctacagcatcgtctct	tccacacacgtggtgaagat
MAFK	human (hsa)	cattaaaggtcaagaaggaggcg	gacatggacaccagctcatca

MYH10	human (hsa)	ctgaggcgctggatctgtg	aaaagcaattgcctcttcagcc
NPPA	human (hsa)	ccgtgagcttcctcctttta	ccaaatggtccagcaaattc
NPPB	human (hsa)	ctccagagacatggatcccc	gttgcgctgctcctgtaac
OTULIN	human (hsa)	acatgtaccgtgctgcagat	aatctcggttctgatgcccc
PFKL	human (hsa)	tttacaagctcctcgcccac	ttcttgcacctgacccttgg
PFKM	human (hsa)	ttcgagacctgcaggcaaat	tccagttcatagccttggcg
PFKP	human (hsa)	aggaacggccagatcgataa	cacctccagaacgaaggtcc
PPFIBP1	human (hsa)	ggtactggcccaaggcaaa	actggagtgggtgatggact
PRNP	human (hsa)	gaccgaggcagagcagtc	agtgttccatcctccaggcttc
RASL11B	human (hsa)	ccggttcctcaccaaacgat	ggctgttctcatggacctgaa
RPL32	human (hsa)	caacgtcaaggagctggaag	tggggttggtgactctgatg
SHROOM3	human (hsa)	ctggagcacggagaaccatta	cacatctctgcgcactacca
SORBS2	human (hsa)	gcctcactcaactccagcat	tctgaggatcggtacagggg
SPSC3	human (hsa)	cgatggaaatggtctcaaggga	ccagcatttggtacgacgtt
Pfkp	rat (rno)	catgaatgctgctgtccgtg	catgccttggtaaccctcgt
Nppb	rat (rno)	cctgcttttccttaatctgtcg	gccatttcctctgacttttctc

Online supplementary Table S3. Primer ISH probes

Target	Forward (fw) & reverse (rv) primer	Cloning strategy
Col3a1	fw: gctctagaggggaatggagcaagacagt	XbaI
	rv: gcgagctcgttcaccagcaacagcagaa	SacI
Nppa	fw: gctctagaagcaaacatcagatcgtgcc	XbaI
	rv: gcgagctcatatgcagagtgggagaggc	SacI
Nppb	fw: gctctagaaggaaatggcccagagacag	XbaI
	rv: gcgagctccaaaagcaggaaatacgctatgt	SacI

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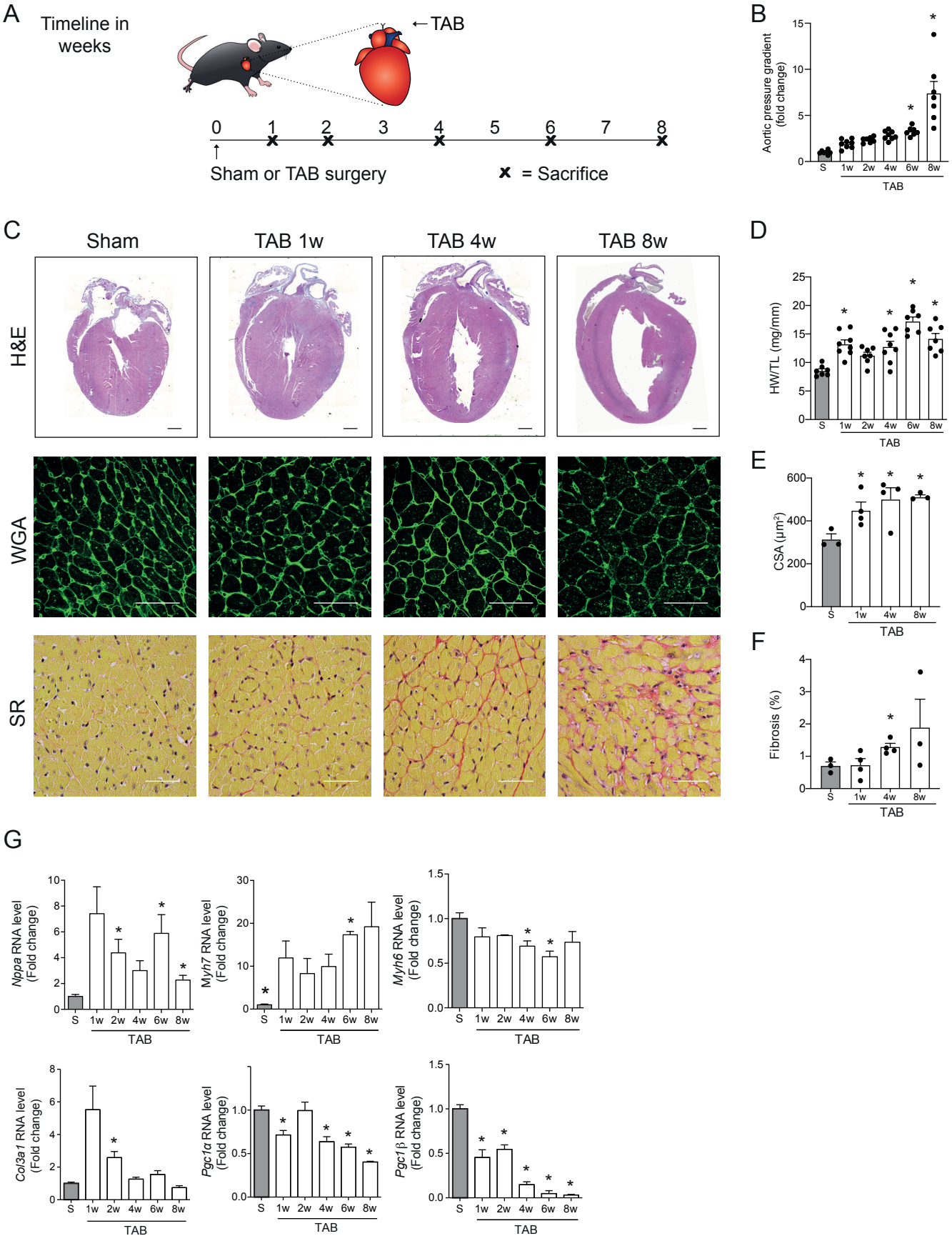
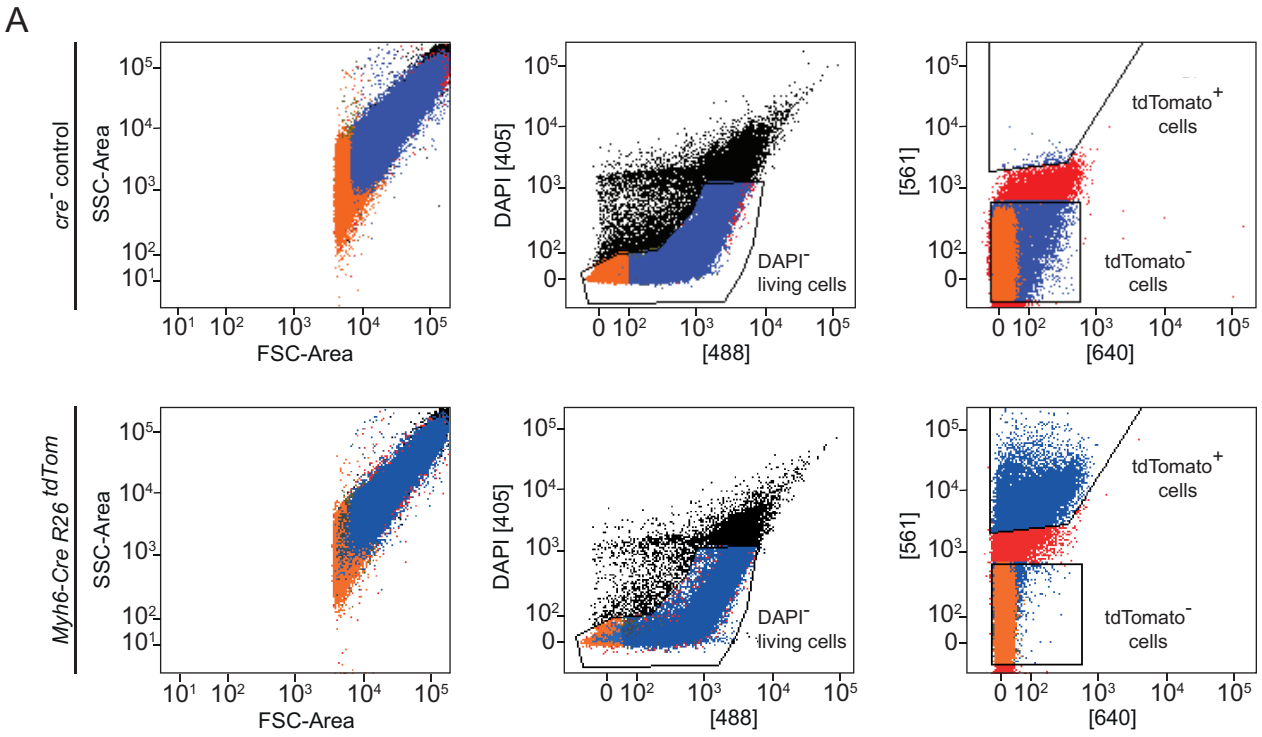
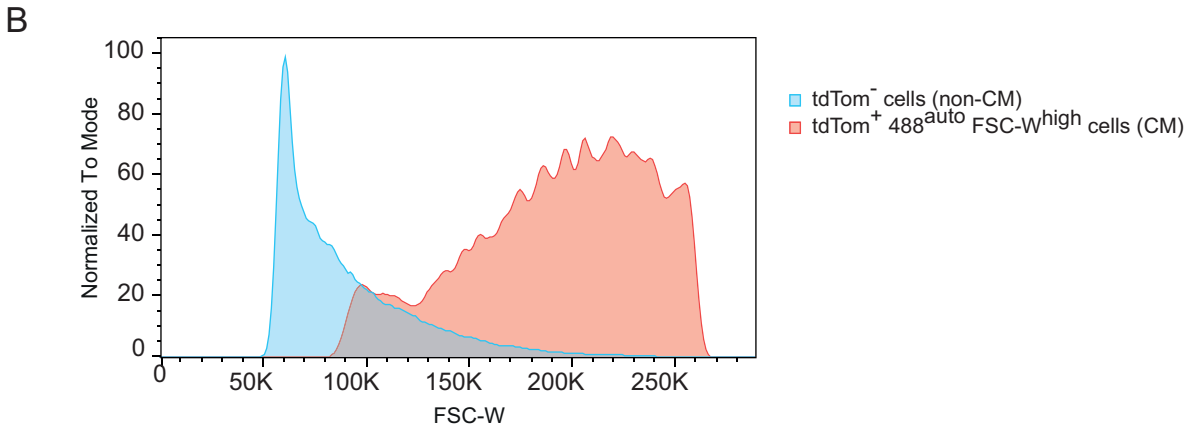
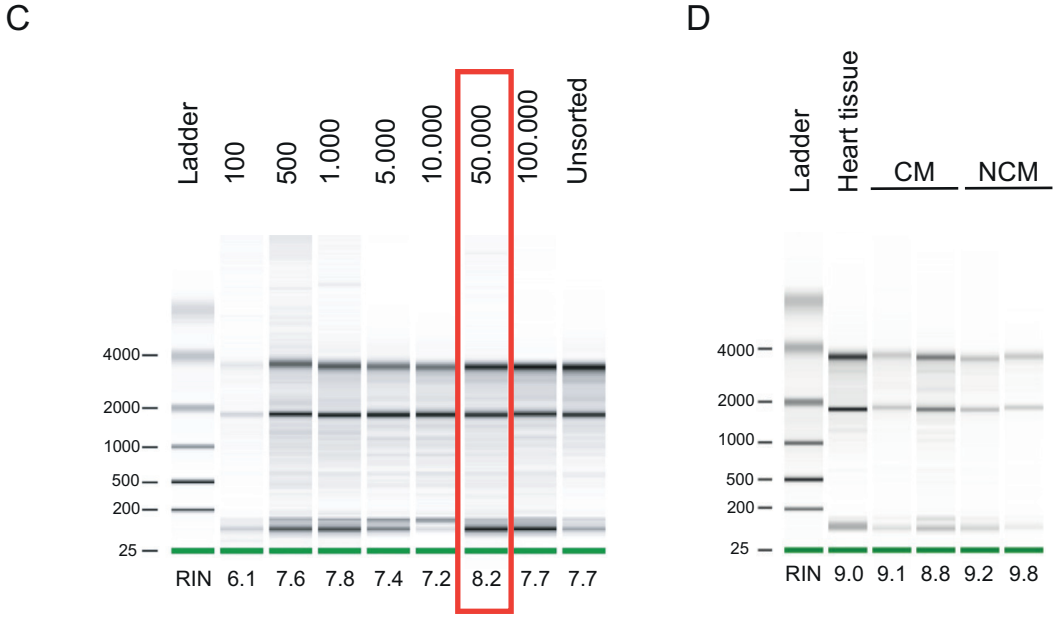
Supplementary Figure S1



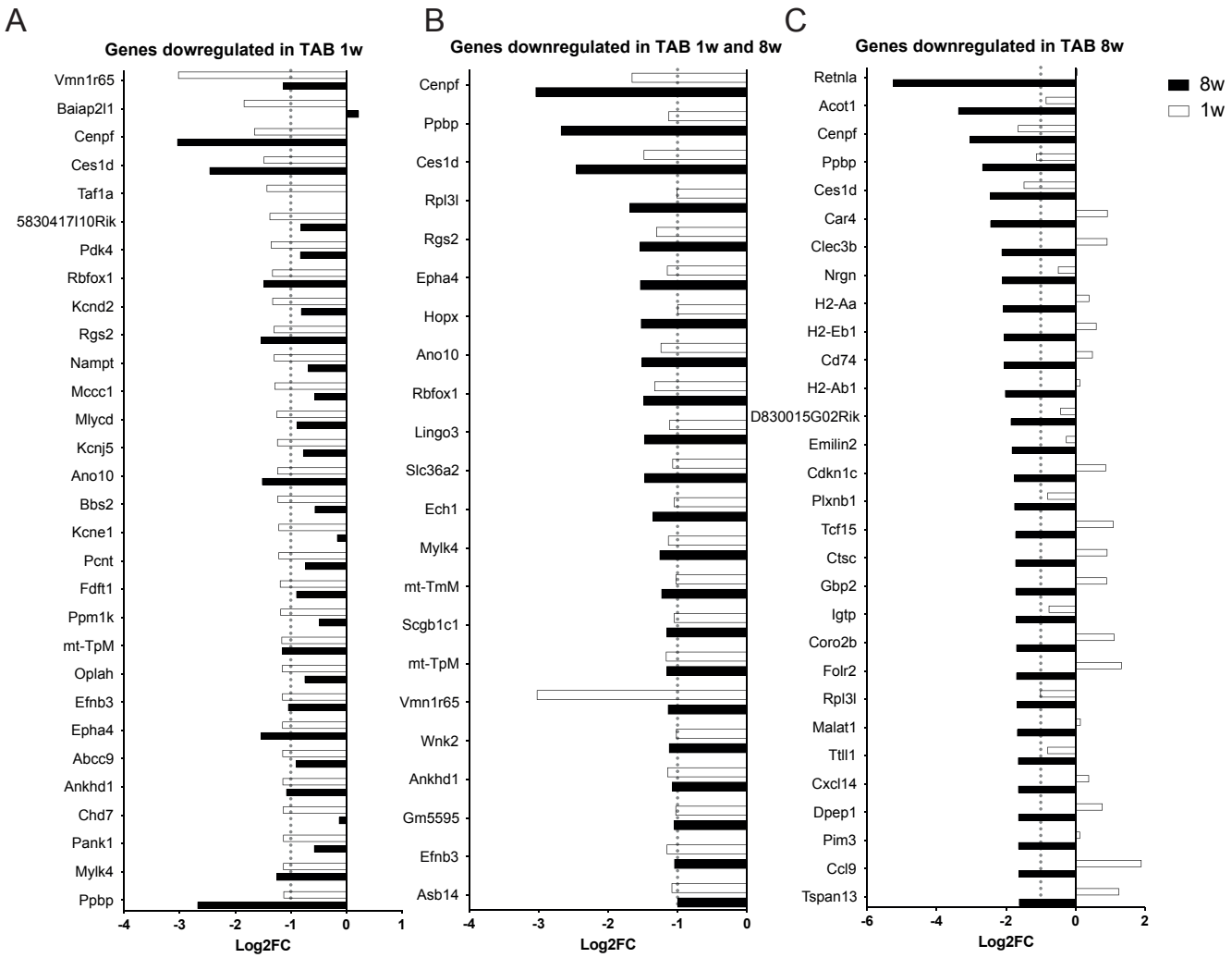
Supplementary Figure S2



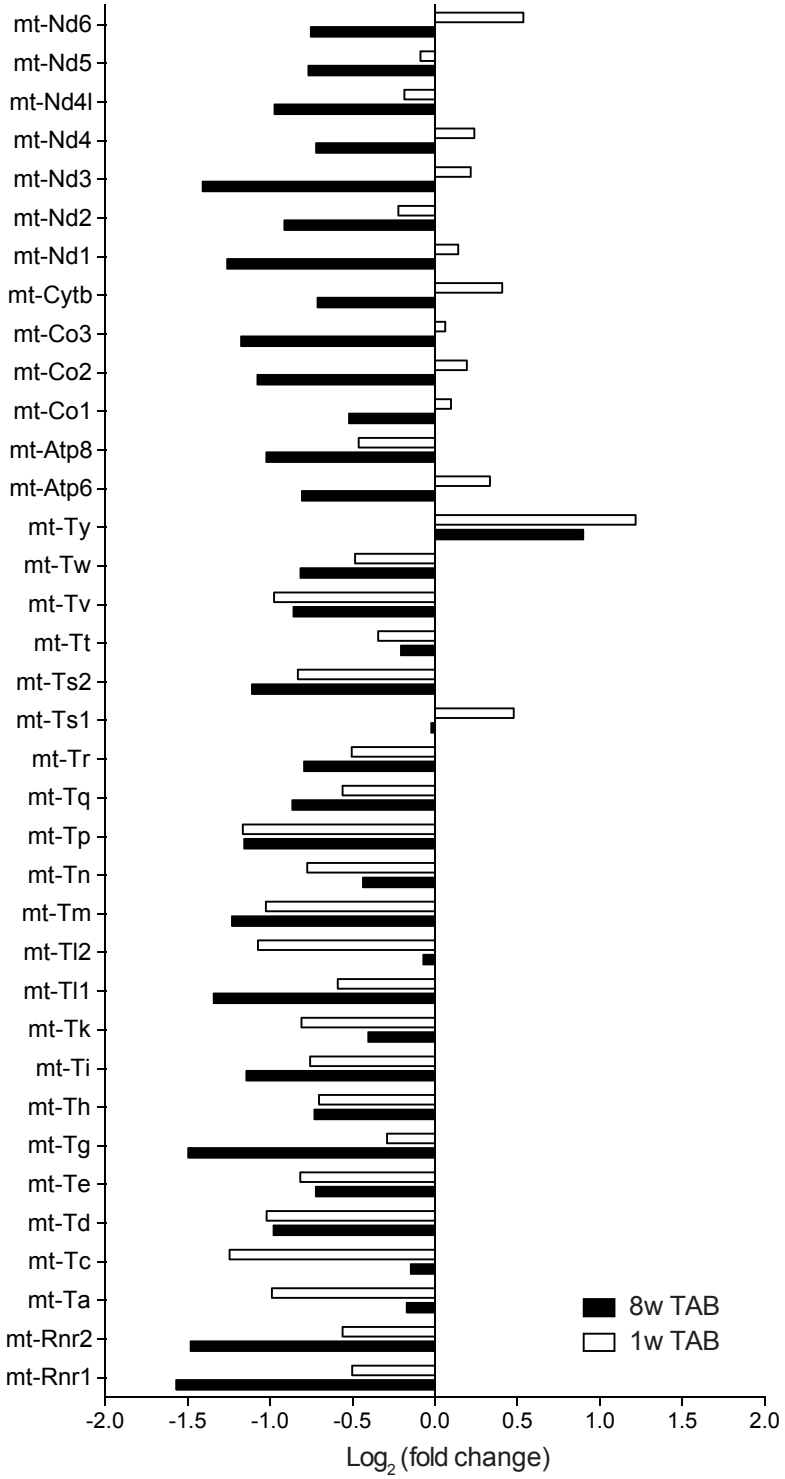




Supplementary Figure S3

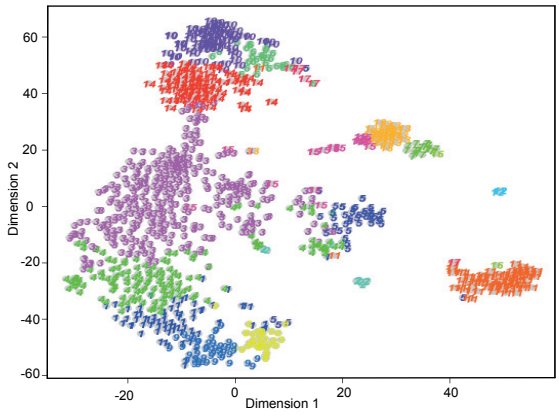


Supplementary Figure S4



Supplementary Figure S5

A



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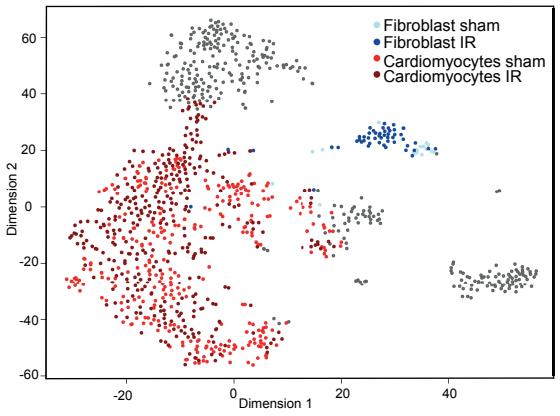

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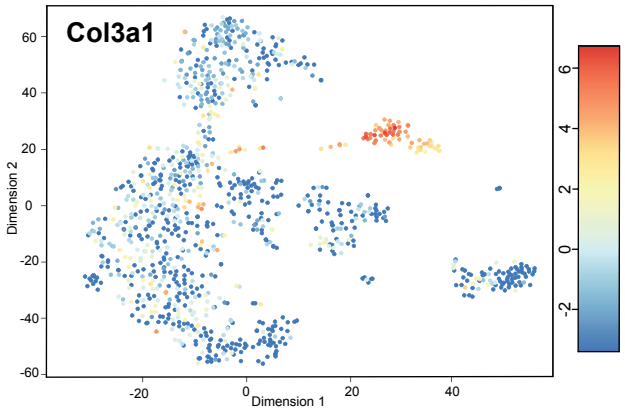

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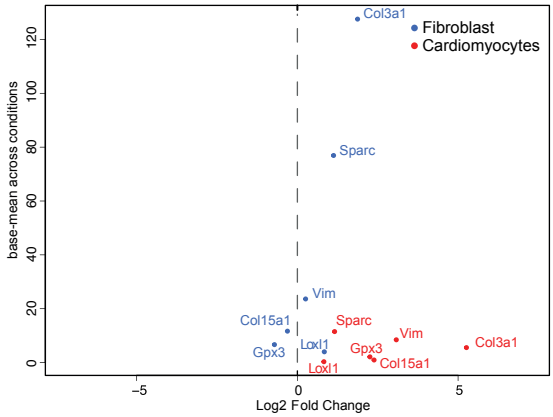

Supplementary Figure S6